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Abstract

Background: Lead exposure during early development causes neurodevelopmental disorders by unknown mechanisms. Epidemiologic studies have focused recently on determining associations between lead exposure and global DNA methylation; however, such approaches preclude the identification of loci that may alter human disease risk.

Objectives: The objective of this study was to determine if maternal, postnatal and early childhood lead exposure alter the differentially methylated regions (DMRs) that control the monoallelic expression of imprinted genes involved in metabolism, growth and development.

Methods: Questionnaire data and serial blood lead levels were obtained from 105 participants (64 females, 41 males) of the Cincinnati Lead Study from birth to 78 months. During adulthood, peripheral blood DNA was used to quantify CpG methylation in peripheral blood leukocytes at DMRs of 22 human imprinted genes using Sequenom EpiTYPER assays. Statistical analyses were conducted using linear regression.

Results: Mean blood lead concentration from birth to 78 months was associated with a significant decrease in *PEG3* DMR methylation, ($\beta=-0.0014$, 95%CI:-0.0023, -0.0005, $p=0.002$), stronger in males, ($\beta=-0.0024$, 95%CI:-0.0038, -0.0009, $p=0.003$) than females ($\beta=-0.0009$, 95%CI:-0.0020, 0.0003, $p=0.1$). Elevated mean childhood blood lead concentration was also associated with a significant decrease in *IGF2/H19* ($\beta=-0.0013$, 95%CI:-0.0023, -0.0003, $p=0.01$) DMR methylation, but primarily in females, ($\beta=-0.0017$, 95%CI:-0.0029, -0.0006, $p=0.005$) than males, ($\beta=-0.0004$, 95%CI:-0.0023, 0.0015, $p=0.7$). Elevated blood lead concentration during the neonatal period was associated with higher *PLAGL1/HYMAI* DMR methylation regardless of sex, ($\beta=0.0075$, 95%CI:0.0018, 0.0132, $p=0.01$). The magnitude of associations between cumulative lead exposure and CpG methylation remained unaltered from 30 to 78 months.

Conclusions: Our findings provide evidence for early childhood lead exposure resulting in sex-dependent and gene-specific DNA methylation differences in the DMRs of *PEG3*, *IGF2/H19* and *PLAGL1/HYMAI* in adulthood.

Introduction

Elevated lead exposure in early life is associated with growth retardation, neurotoxicity, impaired cognitive development in infancy and deficits in attention and executive function (Bellinger et al. 1986; Bellinger et al. 1987). Accumulating evidence also indicates that the effect of lead exposure in early childhood can change neurochemistry (Binns et al. 2007), cause neurobehavioral and cognitive deficits in later life (Finkelstein et al. 1998; Sanders et al. 2009; Winneke et al. 1983; Zahran et al. 2009), decrease brain volume and increase the rate of criminal arrest in adulthood (Cecil et al. 2008; Wright et al. 2008). Although the current actionable level for lead is 5 μ g/dL in the US, the threshold for its toxicity is unknown. Furthermore, the mechanisms by which lead exposure affects diverse neuropathological outcomes is not clearly defined, although epigenetic mechanisms have been proposed (Wright et al. 2010).

Environmental exposures to both physical and chemical agents, especially during early development, can induce alterations in DNA methylation that alter disease susceptibility in adulthood (Bernal et al. 2013; Dolinoy et al. 2006; Dolinoy et al. 2007; Waterland and Jirtle 2003). Animal studies likewise suggest that epigenetic modifications may link lead exposure to neurotoxicity and attention deficit disorders (Faulk et al. 2013; Luo et al. 2014), but stable epigenetic targets responsive to early lead exposure in humans remain uncharacterized.

Because of the relative ease of measuring genomic DNA cytosine methylation at CpG dinucleotides, this endpoint is the most commonly investigated epigenetic modification in epidemiologic studies. *In vitro* and *in vivo* studies demonstrate that DNA methylation is altered by exposure to toxic metals, including arsenic, cadmium and lead (Bolin et al. 2006; Reichard et al. 2007; Takiguchi et al. 2003). The only epigenetic studies conducted in humans thus far, evaluated DNA methylation at *Alu* and *LINE* repeat

elements. They showed associations between maternal patella lead levels and global DNA hypomethylation in newborns (Pilsner et al. 2009) and adult males (Wright et al. 2010). While the biological significance of reduced DNA methylation at repetitive elements in lead-exposed humans is unknown, this epigenetic change also occurs frequently in cancer where it is believed to result in chromosomal instability and genomic mutations (Wilson et al. 2007).

Imprinted genes are characterized by parent-of-origin-dependent monoallelic expression, with the functionally haploid state controlled by differentially methylated regions (DMRs). The inherited imprint methylation marks at these DMRs are established during gametogenesis (i.e. gametic imprints) or early in embryogenesis (i.e. somatic imprints) (Barlow 2011; Reik and Walter 2001). Epigenetic dysregulation of imprinted genes is associated with diseases, including cancer, diabetes, obesity and developmental and neurological disorders (Ishida and Moore 2013; Murphy and Jirtle 2003). DNA methylation marks at imprinted DMRs are generally maintained in tissues from the three germ layers (Murphy et al. 2012b; Waterland et al. 2010; Woodfine et al. 2011). Furthermore, with imprinted genes often occurring in clusters (Edwards et al. 2007) and with the potential for network regulation (Varrault et al. 2006), the methylation status of a single DMR could affect the expression of multiple genes.

The availability of childhood blood lead concentration data and adult peripheral blood DNA from the Cincinnati Lead Study Cohort (Cecil et al. 2008; Dietrich et al. 1987; Dietrich et al. 1993; Dietrich et al. 2001) provided the impetus for the first determination of prenatal and postnatal lead exposure associations with DNA methylation in adulthood for the DMRs of twenty-two imprinted genes as recently described (Skaar et al. 2012).

Material and Methods

Study population. Participants comprised 41 men and 64 women born between 1979 and 1984 who were enrolled in the Cincinnati Lead Study and were successfully re-contacted in 2008-2010. Accrual and lead measurement methods have been described in detail (Cecil et al. 2008; Dietrich et al. 1987). Briefly, pregnant women living in neighborhoods with high prevalence of pediatric lead poisoning were eligible. Women with type 1 or 2 diabetes and neurological, psychiatric or drug addiction disorders were excluded, as were offspring with defects or birth weight <1500 g. Using anodic stripping voltammetry (Roda et al. 1988), lead concentrations were measured in first and second trimester maternal peripheral blood, and blood collected from their children at 10 days of age, every three months for the first 60 months and every 6 months from 60 to 78 months. Umbilical cord blood was not measured due to clotting. During pregnancy, questionnaires were used to collect data on maternal race/ethnicity (Black/White), the number of cigarettes smoked per day, education, occupation, continuous IQ, use of alcohol (Yes/No), marijuana (Yes/No) and narcotics (Yes/No) and children's IQ at age 6.5 years. The protocol was approved by review boards for Cincinnati, Duke, and North Carolina State Universities.

Specimen handling. During the 2008 visit, 108 participants with an average age of 27yrs (25-30 yrs) provided informed consent before peripheral blood specimens for DNA methylation analysis were collected; 105 also had covariable data. Blood was collected in K₂EDTA-treated vacutainer tubes, centrifuged for plasma and buffy coat isolation and shipped to the Jirtle laboratory at Duke University for DNA methylation analysis.

Quantitative methylation analysis. DNA was extracted using Puregene reagents according to manufacturer's protocol (Qiagen, Valencia, CA). Table S1 in Supplemental Materials summarizes DMR

amplicon cleavage fragments, including CpG content and positions. DNA amplification used a touchdown PCR protocol.

Quantitative DNA methylation analysis was performed in two batches using the Sequenom MassARRAY EpiTYPER (Sequenom, San Diego, CA). Primers for human imprinted genes were designed with the use of Epidesigner software (Sequenom, San Diego, CA) to amplify approximately 400 to 600 bp of the imprinted gene DMRs. Cycling conditions for touchdown PCR used are in Table S2 and primers and amplicon data are provided in Table S3. Genomic DNA (2µg) was treated with sodium bisulfite using the EpiTect kit, according to manufacturer's protocols (Qiagen, Valencia, CA). Bisulfite converted DNA (50 ng) was amplified by PCR using HotStarTaq (Qiagen, Valencia, CA).

PCR products were processed by dephosphorylation of unincorporated dNTPs. They were then transcribed *in vitro* with concurrent RNase cleavage using T-cleavage assays according to the manufacturer's standard protocol (Sequenom, San Diego, CA). The transcription reaction was conditioned to remove cations by adding 20 µl H₂O and of 6 mg of Clean Resin (Sequenom, San Diego, CA). Subsequently, the samples were spotted on a 384 pad Spectro-CHIP (Sequenom, San Diego, CA), using a MassARRAY Nanodispenser (Samsung, Irvine, CA), followed by spectral acquisition on a MassARRAY analyzer compact MALDI-TOF MS (Sequenom, San Diego, CA). The percent methylation of CpG sites for each cleavage fragment was determined using EpiTyper software (Sequenom, San Diego, CA).

The Sequenom MassARRAY measured DNA methylation using fragments of reverse-transcribed PCR products, and data output is in CpG "units" in which multiple CpG sites may reside within a single fragment produced by RNase cleavage of transcripts of DMR amplicons (Table S1). The mass difference between fragments with 'T' and 'C' bases (bisulfite converted unmethylated, and unconverted methylated cytosines, respectively) at CpG sites was detected, and the ratio of alternate masses was quantitated to

generate the methylation fraction. The cleavage reaction can produce fragments containing multiple CpG sites. For such fragments, the methylation value was calculated as an average of all sites. Visual inspection of the output from the mass-spectrometer for such fragments with multiple CpGs provided an estimate of the accuracy of this average for individual sites. The detection of primarily two peaks representing hypomethylated and hypermethylated fragments was indicative of strand-specific, *cis*-regulated differential methylation, and the methylation value for the entire fragment was considered to represent each individual CpG site. For most fragments with multiple CpG sites, this two-peak output was the case.

Table S1 summarizes fragments for each amplicon, CpG content and position within the fragments, and shows which fragments were excluded from analysis due to low or high mass outside the detection range, fragment duplication, overlap, or success rate below the 95% threshold. Methylation values for fragments with multiple CpG sites were weighted when included in DMR average methylation and fragments with duplicate masses were included separately, such that the DMR average methylation is the same as if data for each CpG were available.

Reproducibility of 5-10% for MassArray methylation analysis was verified in triplicate for the 22 DMRs using control conceptus tissues representing the three germ layers. In these control fetal tissues, the mean DNA methylation ranged from 45% to 60%. In humans exposed to varying lead levels, the mean DNA methylation ranged from 37% to 74% (Table S3). When more than 5% of samples produced no signals, indicative of errors in spotting or failures in amplification or cleavage, the run was discarded.

Statistical analyses, DNA methylation, and covariable data. A total of 172 CpG-containing cleavage fragments from 22 genomically imprinted gene DMRs had methylation percentages available for the 105 participants. The number of analyzed regions for each DMR and the mean methylation for each DMR

computed from non-missing CpG-containing fragments, are in the last two columns of Table S3. The R-package was used for data analysis (R Core Team 2013).

Lead concentrations ($\mu\text{g/dL}$) were analyzed as age-specific concentrations cumulatively defined as the sum of blood level measurements up to and including the value measured at that age, divided by the number summed (e.g. concentrations at 10 days+3 months+6 months/3=average cumulative concentrations at age 6 months). Lead values were also arrayed and the maximum lead value for each participant was identified. Lead was also categorized into four developmental stages at measurement; prenatal (first or second trimester gestation), neonatal (age 10 days), early childhood coinciding with higher concentrations characteristic of the crawling/oral exploratory developmental window (age 3 to 30 months) and middle childhood coinciding with declining concentrations (age 33 to 78 months). Factors shown to be associated with lead concentrations, from previous analyses of these data (Cecil et al. 2008; Dietrich et al. 1987), and factors known to be associated with DNA methylation were evaluated for confounding in the overall mean and maximum saturated models. Only those with a p-value <0.05 were retained in refined models. Factors evaluated for confounding were maternal education, smoking and race, as well as offspring sex and batch. Only sex (male/female), batch (first or second), and smoking (none, ≤ 0.5 , 1, 1.5 and 2 packs per day, computed from the number of cigarettes smoked daily, assuming 20 cigarettes in a pack) remained significant, and were retained in refined models.

Because some DNA methylation values were not normally distributed, the \log_2 of the standardized regression coefficients, (M-values= $[\log_2(\frac{\text{Beta Value}}{1-\text{Beta Value}})]$), were used in adjusted linear regression models. These were compared to unstandardized regression coefficients, and the results were similar (data not shown). For ease of interpretation, we present unstandardized regression coefficients with 95%

confidence intervals in tables, while regression coefficients at each lead measurement are plotted without confidence intervals. A p -value ≤ 0.05 was considered statistically significant.

The limited sample size precluded adjustment for multiple comparisons. Instead, we included only DMRs for which the level of methylation of more than 3 CpG-containing fragments were correlated $r > 0.8$, suggesting *cis*-acting regulation (data not shown). The CpG-containing fragments also had to have a persistent significant ($p < 0.05$) associations with lead exposure for any four consecutive mean lead measurements (e.g. 1 year association were significant and in the same direction for lead levels measured at 3, 6, 9 and 12 months) as seen in Figure 1.

Results

Study participants, lead concentrations and DNA methylation. The majority (90%) of participants were born to Black women, ~70% had less than a high school education, and the median IQ was 75 (Table 1). While ~50% of participants were exposed to cigarette smoke *in utero*, exposure to alcohol, marijuana and narcotics was uncommon (<13%). Prenatal mean blood lead concentration was $9.1 \mu\text{g/dL}$ ($\text{sd}=6.0 \mu\text{g/dL}$), postnatal mean lead level was $13.6 \mu\text{g/dL}$ ($\text{sd}=5.5 \mu\text{g/dL}$), and the maximum mean lead level was $25.3 \mu\text{g/dL}$ ($\text{sd}=5.3 \mu\text{g/dL}$). Blood lead levels in males and females were comparable throughout the study visits; the concentration increased rapidly with age, peaking at age 20-25 months, and then decreased (Figure 2). Lead concentrations and the distribution of covariable data were comparable with those who were successfully contacted again in 2008-2010 ($p \geq 0.14$).

Data for 37 of 172 CpG-containing fragments were missing for >5% of participants, leaving 135 fragments for analysis. The mean DMR methylation levels for the 105 participants exposed to varying levels of lead ranged from 37% to 74% (Table S3).

Associations between early lead exposure and DMR methylation. Lead concentrations were highly correlated within individuals, especially in early childhood (Table S4). Of the 22 DMRs, those for 6 imprinted genes had more than 3 CpGs with correlation >0.8 - *PEG10*, *DLK1/MEG3*, *DIRAS*, *PEG3*, *IGF2/H19*, and *PLAGL1/HYMAI*. However, the mean and maximum lead concentrations were only persistently associated with the DNA methylation of DMRs for *PEG3*, *IGF2/H19* and *PLAGL1/HYMAI* DMRs (Table 2, Table S5 and Figure 3).

Childhood lead levels and *PEG3* DMR methylation. Table 2 shows an association between mean lead concentrations across childhood and lower *PEG3* DMR methylation in adulthood ($\beta=-0.0014$, 95% CI:-0.0023, -0.0005, $p=0.002$). These associations were primarily in males ($\beta=-0.0024$, 95% CI:-0.0038, -0.0009, $p=0.003$) compared to females ($\beta=-0.0009$, 95% CI:-0.0020, 0.0003, $p=0.1$). The cross-product term p -value for mean lead concentrations and sex was 0.09. This association in males corresponds to a 0.24% decrease in CpG methylation at the *PEG3* DMR, for every 1 $\mu\text{g}/\text{dL}$ increase in lead concentration. There were statistically significant inverse associations between *PEG3* DMR methylation and lead concentrations in early ($\beta=-0.0012$, 95% CI:-0.0020, -0.0004, $p=0.005$) and middle childhood ($\beta=-0.0013$, 95% CI:-0.0021, -0.0005, $p=0.002$). The associations were significant only in males during early ($\beta=-0.0023$, 95% CI:-0.0036, -0.0009, $p=0.002$ for males and $\beta=-0.0006$, 95% CI:-0.0016, 0.0005, $p=0.3$ for females) and middle childhood ($\beta=-0.0018$, 95% CI:-0.0031, -0.0004, $p=0.02$ for males and $\beta=-0.0009$, 95% CI:-0.0020, 0.0001, $p=0.1$ for females). Cross-product terms for early and middle childhood lead exposure and sex were $p=0.04$ and 0.3, respectively. There were no statistically significant associations between prenatal or neonatal lead concentration and *PEG3* DMR methylation, although the direction of associations was largely similar to those of early and middle childhood.

Patterns of association observed in overall and sex-specific mean lead concentrations were also evident when maximum lead concentrations were considered (Table S5). The association between the maximum lead concentration and decreased *PEG3* DMR methylation in adulthood ($\beta=-0.0007$, 95% CI:-0.0012 - 0.0003, $p=0.003$) was also more apparent in males ($\beta=-0.0013$, (95% CI:-0.0021, -0.0006, p -value=0.001) than in females ($\beta=-0.0004$, 95% CI:-0.0010, 0.0002, $p=0.3$). Maximum lead levels in the prenatal and neonatal ages were too low for meaningful analyses.

To refine potential windows of vulnerability, non-standardized regression coefficients were plotted for the associations between DMR methylation and averaged cumulative lead concentrations for each age at which lead was measured (Figure 3). Figure 3A confirms the association in Table 2 that higher lead exposure is associated with lower *PEG3* DMR methylation, and also shows that this association does not vary significantly after age 12-20 months to age 6.5 years, despite wide variation in lead concentrations during the observation period. These associations are male-specific.

Childhood lead levels and *IGF2/H19* DMR methylation. Regression coefficients and p-values for the association between mean lead concentration and *IGF2/H19* DMR methylation in adulthood are also shown in Table 2. Mean childhood lead concentration was significantly associated with adult *IGF2/H19* DMR methylation ($\beta= -0.0013$, 95% CI:-0.0023, -0.0003, $p=0.01$). This association may be stronger in females ($\beta=-0.0017$, 95% CI:-0.0029, -0.0006 $p=0.005$) than males ($\beta=-0.0004$, 95% CI:-0.0023, 0.0015, $p=0.7$).

Associations for lead exposure and *IGF2/H19* DMR methylation were also found in early ($\beta=-0.0016$, 95% CI:-0.0027, -0.0005, $p=0.007$ and middle childhood ($\beta=-0.0016$, 95%CI:-0.0027, -0.0004, $p=0.008$) in females. These associations were weaker and less consistent in males, for early ($\beta=0.0004$, 95% CI:-0.0014, 0.0022, $p=0.7$) and middle childhood ($\beta=-0.0009$, 95%CI:-0.0026, 0.0008, $p=0.3$). The p-values

for cross-product terms for early and middle childhood lead levels and sex were 0.10 and 0.6, respectively. This age and sex-specific pattern of association was also noted when maximum blood lead concentrations were evaluated (Table S5). As with the *PEG3* DMR methylation, associations with prenatal and neonatal exposure were weaker although in the same direction.

Further exploration of cumulative lead concentrations suggests a female-specific association between lead exposure and lower methylation at the *IGF2/H19* DMR. The magnitude of the association between cumulative lead concentration and *IGF2/H19* DMR methylation was the same after 12-20 months (Figure 3B). As with *PEG3* DMR, this similarity persists for the entire observation period.

Childhood lead Levels and *PLAGL1/HYMAI* DMR methylation. Unlike *IGF2/H19* and *PEG3* which showed no evidence for association between neonatal lead exposure and DMR methylation, *PLAGL1/HYMAI* DMR methylation was positively associated with lead concentrations during this period ($\beta=0.0075$, 95% CI:0.0018, 0.0132, $p=0.01$). The wide confidence intervals suggest that this association varied little between males ($\beta=0.0074$, 95%CI:-0.0001, 0.0150, $p=0.06$) and females ($\beta=0.0030$, 95%CI:-0.0069, 0.0129, $p=0.6$). No significant association was found between lead exposure and *PLAGL1/HYMAI* DMR methylation at any other age. Refined age-dependent and sex-specific analyses suggest that the magnitude of associations remained unaltered after ~12-20 months to the end of the observation period (Figure 3C).

Discussion

The DMRs regulating monoallelic expression of imprinted genes are proposed to function as epigenetic archives of early exposure to environmental factors (Hoyo et al. 2009). Nevertheless, until now, no empirical data have demonstrated associations between early exposure to lead and adult CpG methylation at DMRs controlling the parent-of-origin silencing of imprinted genes. Environmentally-induced DNA

methylation changes at imprint DMRs are usually stable once established (Heijmans et al. 2008), and have been associated with common chronic diseases and conditions, including neurological disorders, obesity, type-2-diabetes, and some cancers (Azzi et al. 2013; Feinberg 2007; Hoyo et al. 2012; Ishida and Moore 2012).

We have undertaken an analysis of 22 DMRs regulating human imprinted genes, and evaluated relationships between DNA methylation in adulthood and lead exposure spanning from the first or early second trimester to age 6.5 years. Our key findings were that childhood lead exposure was associated with significantly lower DNA methylation levels at the DMR regulating *PEG3*. We also found modest but consistent associations between average lead concentration and decreased methylation of the *IGF2/H19* DMR, and higher DNA methylation levels at the *PLAGL1/HYMAI* DMR in relation to neonatal exposure. These data further indicated that while childhood lead exposure was associated with differences in *PEG3* DMR methylation in males and the *IGF2/H19* DMR methylation in females, the association between neonatal lead concentrations and *PLAGL1/HYMAI* DMR methylation may not be sex-specific. Notably, lead associations with DNA methylation of imprint regulatory elements at these three loci were found in lead measured before age 30 months, regardless of sex or DMR. These data support the contention that environmentally-driven perturbations at these DMRs occurs early. Furthermore, developmental differences between the sexes may dictate the patterns of gene regulation that ensue in response to early challenges with this heavy metal.

PEG3 DMR methylation and early lead exposure. Although childhood lead exposure has been associated with increased risk of neurodevelopmental disorders (Dietrich et al. 2010), the mechanisms underlying these pathological conditions are poorly understood. *PEG3* plays a critical role in brain development, with expression mainly in the mesencephalon and pituitary gland; in the adult brain *PEG3*

is primarily found in the hypothalamus and the pituitary gland (Li et al. 1999). In mouse models, *Peg3* also plays an important role in social and maternal nurturing behaviors, and paternal transmission of disrupted *Peg3* also leads to restricted growth (Chiavegatto et al. 2012; Li et al. 1999). In humans, hypermethylation at this locus has been associated with decreased gene expression of this tumor suppressor gene in cervical (Nye et al. 2013) and ovarian (Feng et al. 2008) cancers. In primary neuronal cell cultures derived from wild-type, p53-deficient, or Bax-deficient mice, overexpression of *Peg3* led to decreased neuronal viability via *p53* and *Bax* dependent pathways (Johnson et al. 2002). It is therefore possible that the male specific reduced brain volume recently observed in these study participants (Cecil et al. 2008) may result, in part, from the dysregulation of *PEG3* during early development.

Interestingly, early childhood but not prenatal or neonatal lead concentrations were associated with adulthood *PEG3* DMR hypomethylation, an association that may be specific to males. As these DNA methylation marks are established early, it is possible that methylation differences observed were due to lead accumulated *in utero* and mobilized from soft tissue and bone after birth together with concurrent exposure. Alternatively, the reduced DNA methylation of the *PEG3* DMR marks may have been established postnatally (Loke et al. 2013). The latter possibility is consistent with human developmental studies suggesting that the first 1000 days can dictate life-time risk of common diseases (Victora et al. 2008). Discriminating between these possibilities requires larger studies with long-term follow-up.

IGF2/H19 imprinted domain and early lead exposure. The paternally expressed *insulin-like growth factor-2* (*IGF2*) is a commonly studied imprinted gene, and is frequently shown to be altered epigenetically by *in utero* environmental perturbations, and in cancer (Cruz-Correa et al. 2004; Cruz-Correa et al. 2009; Cui et al. 2003; Heijmans et al. 2008; Hoyo et al. 2011; Hoyo et al. 2012; Murphy et al. 2012a). Dysregulation of the *IGF2/H19* domain was initially associated with Beckwith-Wiedemann

syndrome (BWS) (Engel et al. 2000). Decreased DNA methylation at the *IGF2/H19* DMR has been associated with reduced *IGF2* expression in bladder cancer (Takai et al. 2001). This occurs when enhanced binding of the CTCF insulator protein to the normally unbound paternal allele (Nakagawa et al. 2001) blocks promoter interactions with downstream enhancers, thereby reducing gene expression (Hark et al. 2000; Kanduri et al. 2000). *Igf2* over-expression results in animal overgrowth (Sun et al. 1997), whereas gene repression results in restricted growth (DeChiara et al. 1990). IGF2 is also required for memory formation (Chen et al. 2011).

PLAGL1/HYMA1 imprinted domain and early lead exposure. A higher order regulation of imprinted gene clusters is thought to exist and occur through epigenetic marks present at imprinting centers (Lewis and Reik 2006). Our finding that neonatal lead exposure is associated with increased methylation at the *PLAGL1/HYMA1* (*ZAC*) DMR regardless of sex is potentially of biological importance. In animals, microarray analysis shows that knockout of the mouse homolog, *Zac1* (*Plagl1*), disrupts a network of coordinately regulated genes containing a large number that are also imprinted (Varrault et al. 2006). *In vitro* studies show induction of imprinted *Igf2*, *Cdkn1c*, *H19*, *Dlk1* and *Mest* when *Zac1* is overexpressed (Varrault et al. 2006). Conversely, loss of *Zac1* expression in null mice results in inhibition of *Igf2*, *Cdkn1c*, *H19* and *Dlk1* expression. Another imprinted gene network was identified by analyzing chromatin domains in other regions of the genome that interact with the *Igf2/H19* domain, *in vitro* (Zhao et al. 2006). The maternally expressed long noncoding *H19* RNA and the methyl-CpG-binding protein Mbd1 form a complex that regulates multiple imprinted genes by interacting with histone lysine methyltransferases. In mice, paternally expressed *Plagl1* is implicated in transient neonatal diabetes when over-expressed (Ma et al. 2004). In ovarian cell lines, *PLAGL1* was found to regulate *CDKN1C* (*p57^{KIP2}*) expression and cell growth by inducing *LIT1* transcription in a methylation-dependent manner (Arima et al. 2005). Overexpression of *PLAGL1* induced *IGF2*, *H19* and *CDKN1C* expression in a prostate cancer

cell line (Ribarska et al. 2014). Together, these studies support a set of imprinted genes functioning in a network, coordinated in part by *Zac* (Finkelstein et al. 2009; Lui et al. 2008). Thus, environmentally-induced epigenetic shifts of the *PLAGL1/HYMAI* regulatory DMR have the potential to alter network-wide imprinted gene expression. Studies with a larger number of DMRs are required to clarify the role of the *PLAGL1/HYMAI* DMR in the higher order regulation of imprint clusters. Nevertheless, our findings are consistent with the idea that the far-reaching effects of early lead exposure may be mediated by stable, mitotically heritable epigenetic alterations in DMRs controlling imprinted gene expression.

A cautious interpretation of our findings is warranted. While lead is known to target multiple organs, DNA methylation was measured using unfractionated peripheral blood collected in adulthood, the only accessible cell type, raising concerns about potential confounding by cell type, and other exposures during the life course that could not be evaluated. Another limitation of this study is the relatively small sample size which reduced the precision of associations found. Assay limitations also precluded the measurement of DNA methylation for ~50% of CpGs within CpG-containing fragments. Since methylation values for CpG-containing fragments were averaged from individual CpG sites with similar methylation values and are *cis*-acting, such missing data should not alter our findings. The small amount of peripheral blood leukocyte DNA available for methylation analysis also precluded the determination of altered gene expression via other epigenetic mechanisms (e.g. histone modifications and chromatin structure changes); however, similar methylation changes at both the *IGF2/H19* and *PEG3* DMRs have been associated previously with altered gene expression in human cancers (Cui et al. 2003; Nye et al. 2013). Thus, our findings add preliminary support to accumulating evidence indicating that early lead exposure and gene-specific, epigenetic dysregulation of some imprinted gene DMRs may contribute to developmental abnormalities (Ishida and Moore 2013).

Our study also has major strengths. They include the determination of lead levels ~30 years prior to quantification of DNA methylation levels at imprinted gene DMRs. The numerous measurements of lead concentration during early development also facilitate estimating developmental windows in which lead exposure may exert its effects on regulatory DMRs. Furthermore, blood lead concentrations reflect both short- and longer-term exposure, including lead mobilized from physiological deposits.

To our knowledge, our findings represent the first attempt in humans, to quantify associations between early lead exposure, and DNA methylation alterations in adulthood at imprinted loci that are known experimentally, to result in developmental and neurological disorders if perturbed early in development. Because lead exposure, disproportionately affects the lower socioeconomic strata (Emerson 2012; Rai et al. 2012; Wright et al. 2008), if replicated in larger studies, our findings may offer a potential explanation for observed DNA methylation differences among socioeconomic strata (Szyf 2012; Szyf 2013).

Conclusions

Preventing lead exposure during vulnerable developmental windows remains sound policy. Nevertheless, effective therapeutic and public health strategies will depend upon a better understanding of mechanisms underpinning the associations between lead exposure and the genesis of neurodevelopmental disorders, and other poor health outcomes. Improved understanding should also guide policy regarding the highest tolerable limits in humans, a value currently unknown. While the small sample size limits inference, this study provides preliminary evidence for significant associations between early lead exposure and DNA methylation at the regulatory regions of *PEG3*, *H19/IGF2* and *PLAGL1/HYMAI*. Because these changes in the epigenome are acquired early, resultant shifts in the regulation of imprinted genes may contribute to increased risk of poor health outcomes (Ishida and Moore 2013; Murphy and Jirtle 2003). It remains unknown whether lead exposure previously associated with decreased gray matter volume (Cecil et al.

2008) and delinquent behavior (Dietrich et al. 2001) reported in this study population is mediated in part by the epigenetic alterations in imprinted gene regulatory elements, but this intriguing possibility needs to be investigated.

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Table 1. Characteristics of study participants

CHARACTERISTIC	n (%)	Males (n=41)	Females (n=64)
Race			
White	10 (9.5)	5 (12.2)	5 (7.8)
Black	95 (90.5)	36 (87.8)	59 (92.2)
Education			
≤High School	71 (67.7)	33 (80.5)	38 (59.3)
>High School	34 (32.4)	8 (19.5)	26 (40.6)
Range (yr)	6 -16	9-16	6-16
Maternal Alcohol Use			
Yes	13 (12.4)	4 (9.8)	9 (14.1)
No	92 (87.6)	37 (90.2)	55 (85.9)
Maternal Narcotic Use			
Yes	1 (1.5)	0 (0.0)	1 (1.6)
No	104 (98.5)	40 (100.0)	63 (98.4)
Maternal Marijuana Use			
Yes	11 (10.5)	4 (9.8)	7 (10.9)
No	94 (89.5)	37 (90.2)	57 (89.1)
Maternal Tobacco Use During Pregnancy			
None	48 (45.7)	23 (56.1)	25 (39.1)
≤pack of less per day	43 (41.0)	13 (31.7)	30 (40.6)
1 pack per day	11 (10.5)	3 (7.2)	8 (12.5)
1.5 packs a day	2 (1.9)	1 (2.4)	1 (1.6)
2 packs per day	1 (1.0)	1 (2.4)	0 (0)
Birth Weight (g)	3096 ^a (1990 - 4340) ^b	3184 (2000-4260)	3040 (1990-4340)
Maternal IQ	75 ^a (58 -102) ^b	74 (61-97)	76 (58-102)
Participant IQ	87 ^a (50 -116) ^b	87 (50-111)	88 (67-116)
Lead concentrations during developmental windows (μg/L)			
Childhood (birth to 78 months) mean	13.6 (5.5)	13.7 (5.5)	13.5 (5.5)
Neonatal period (≤28 days)	14.5 (5.9)	14.5 (6.1)	14.5 (5.7)
Early childhood (age ≤30 months)	14.5 (5.9)	14.5 (6.1)	14.5 (5.7)
Middle childhood (>30-78 months)	13.0 (6.1)	13.2 (5.9)	12.8 (6.2)
Age at blood draw for DNA methylation determination	26.7 (25.4-29.6)	26.9 (25.5-29.6)	26.5 (25.4-28.4)

^a Median, ^b Range. ^cPacks per day is based on a typical American package of 20 cigarettes.

Table 2. ^{a,b}Regression coefficients for the association between mean lead exposure and *PEG3*, *IGF2/H19*, and *PLAGL1/HYMAI* DMR methylation

DMR/CpG	*Both sexes	Males	Females
lead exposure	coef (95%CI), p-value	coef (95%CI), p-value	coef (95%CI), p-value
<i>PEG3</i>			
Mean life childhood lead levels (birth to age 78 months)	-0.0014 (-0.0023, -0.0005), 0.002	-0.0024 (-0.0038, -0.0009), 0.003	-0.0009 (-0.0020, 0.0003), 0.1
Prenatal lead levels (2 nd or 3 rd trimester)	-0.0011 (-0.0028, 0.0005) 0.2	0.00001 (-0.0028, 0.0028) 1.0	-0.0017 (-0.0037, 0.0004) 0.1
Neonatal lead levels (age 10 days)	-0.0010 (-0.0025, 0.0004), 0.2	-0.0011 (-0.0030, 0.0007), 0.2	-0.0010 (-0.0035, 0.0016), 0.5
Early childhood levels (age 3 – 30 months)	-0.0012 (-0.0020, -0.0004), 0.005	-0.0023 (-0.0036, -0.0009), 0.002	-0.0006 (-0.0016, 0.0005), 0.3
Middle childhood levels (age 33 – 78 months)	-0.0013 (-0.0021, -0.0005), 0.002	-0.0018 (-0.0031, -0.0004), 0.02	-0.0009 (-0.0020, 0.0001), 0.1
<i>IGF2/H19</i>			
Mean life childhood lead levels (birth to age 78 months)	-0.0013 (-0.0023, -0.0003), 0.01	-0.0004 (-0.0023, 0.0015), 0.7	-0.0017 (-0.0029, -0.0006), 0.005
Prenatal lead levels (2 nd or 3 rd trimester)	-0.0004 (-0.0022, 0.0014) 0.7	0.0007 (-0.0025, 0.0040) 0.7	-0.0011 (-0.0033, 0.0011) 0.3
Neonatal lead levels (age 10 days)	-0.0013 (-0.0029, 0.0003), 0.1	-0.0009 (-0.0032, 0.0013), 0.4	-0.0025 (-0.0052, 0.0002), 0.08
Early childhood levels (age 3 – 30 months)	-0.0009 (-0.0019, 0.00001), 0.06	0.0004 (-0.0014, 0.0022), 0.7	-0.0016 (-0.0027, -0.0005), 0.007
Middle childhood levels (age 33 – 78 months)	-0.0013 (-0.0022, -0.0004), 0.005	-0.0009 (-0.0026, 0.0008), 0.3	-0.0016 (-0.0027, -0.0004), 0.008
<i>PLAGL1/HYMAI</i>			
Mean life childhood lead levels (birth to age 78 months)	0.0016 (-0.0021, 0.0052), 0.4	0.0023 (-0.0046, 0.0091), 0.5	-0.0001 (-0.0045, 0.0044), 1.0
Prenatal lead levels (2 nd or 3 rd trimester)	-0.0023 (-0.0091, 0.0044) 0.5	-0.0041 (-0.0160, 0.0078) 0.5	-0.0023 (-0.0104, 0.0058) 0.6
Neonatal lead levels (age 10 days)	0.0075 (0.0018, 0.0132), 0.01	0.0074 (-0.0001, 0.0150), 0.06	0.0030 (-0.0069, 0.0129), 0.6
Early childhood levels (age 3 – 30 months)	0.0024 (-0.0011, 0.0059), 0.2	0.0055 (-0.0006, 0.0115), 0.08	0.00001 (-0.0042, 0.0042), 1.0
Middle childhood levels (age 33 – 78 months)	0.0006 (-0.0028, 0.0040), 0.7	-0.0009 (-0.0071, 0.0053), 0.8	-0.0001 (-0.0043, 0.0042), 1.0

^a Unstandardized regression coefficients. All models adjusted for batch (first or second) and maternal cigarette smoking (none, one half, 1 and 2 packs a day). Models of combined estimates for males and females are also adjusted for sex.

^b Mean for each developmental period (early childhood) was derived by summing up lead levels for the participant and dividing by the number of observations.

Figure Legends

Figure 1: Number of consecutive ages with significant ($p \leq 0.05$) association of DMR methylation with lead exposure from birth to age 78 months. The dashed line indicates the threshold for imprinted gene inclusion.

Figure 2: Mean postnatal circulating lead levels in males (open circles, dashed line) and females (closed circles, solid line) versus childhood age. (Shaded, Early Childhood-10 days to 30 months; Unshaded, Middle Childhood-33 to 78 months)

Figure 3: Unstandardized regression coefficients for associations between DMR methylation for A) *PEG3*, B) *IGF2/H19* and C) *HYMA/PLAGL1* and the average cumulative lead level (obtained by summing up blood level measurements up to, and including the value measured at that age, divided by the number summed (Figures 3A, 3B, 3C), in males (open circles) and females (closed circles) (Shaded, Early Childhood; Unshaded, Middle Childhood)

Figure 1.

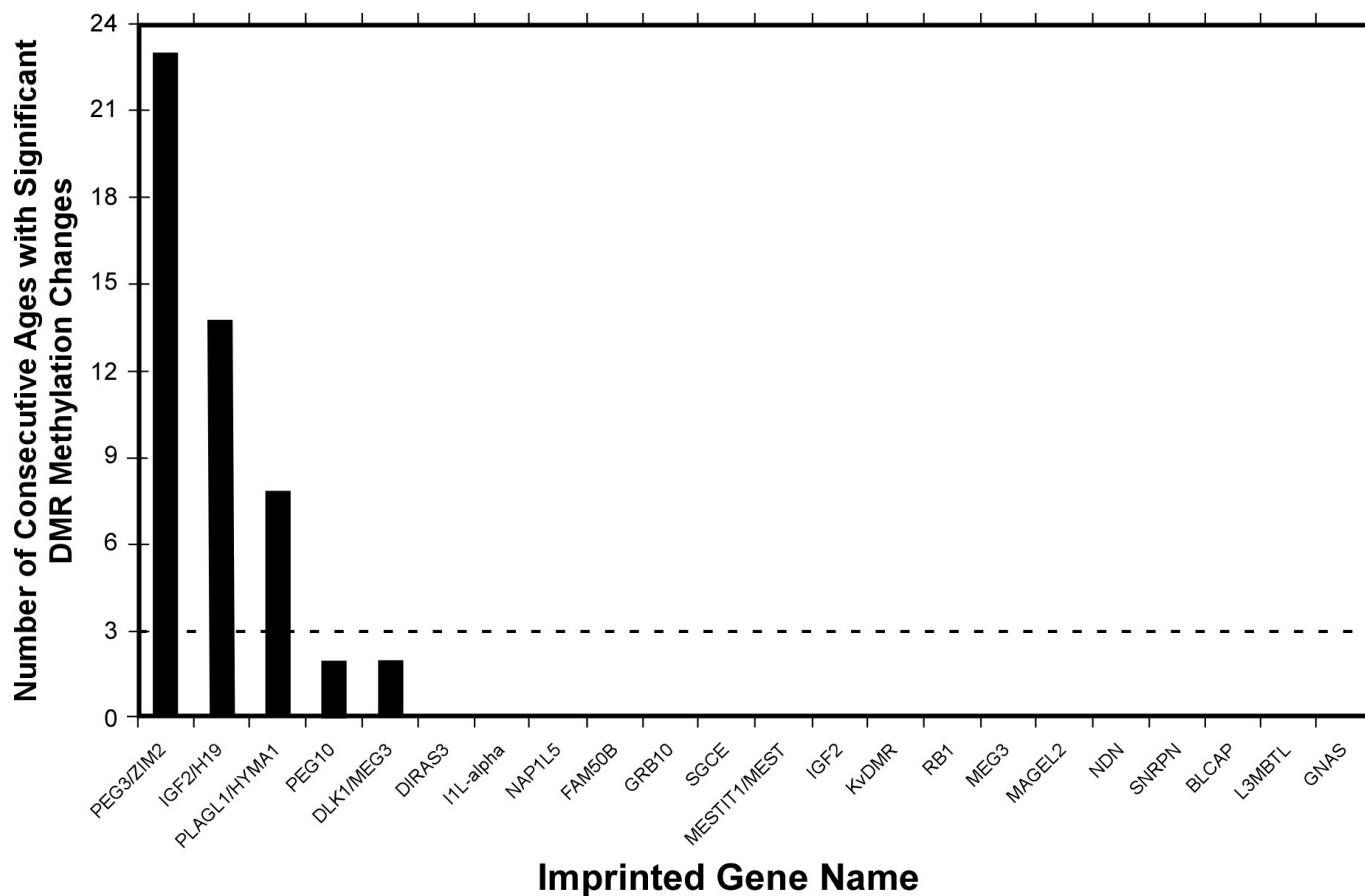


Figure 2.

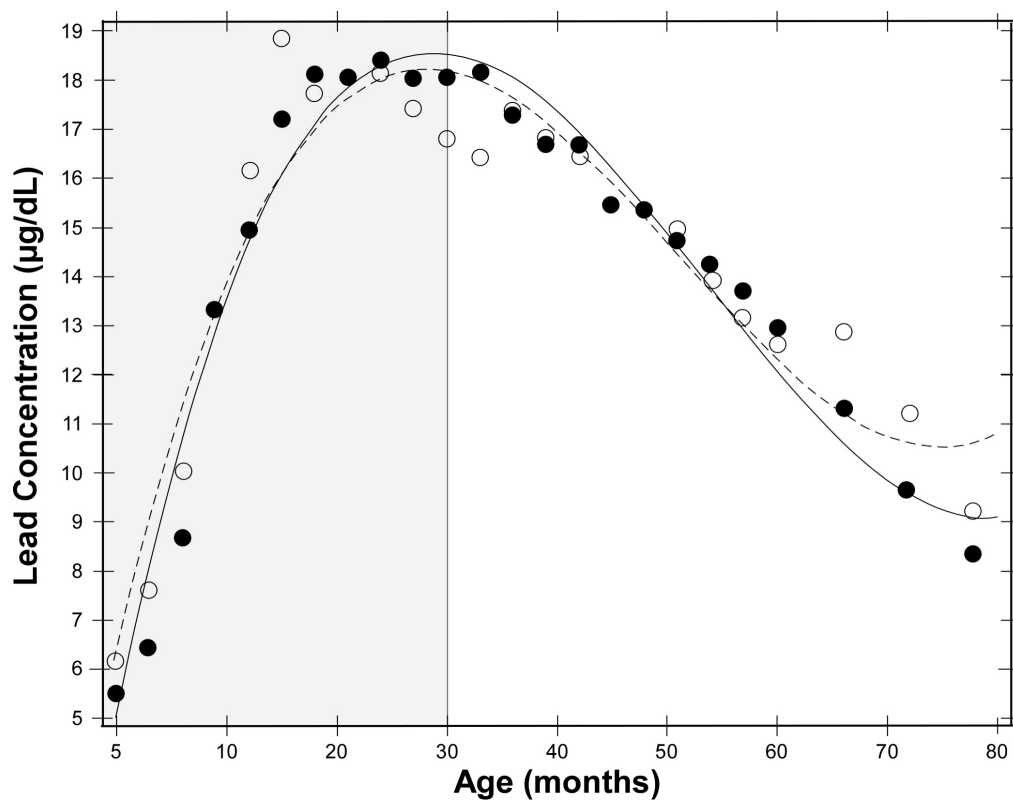


Figure 3.

